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Human muscle precursor cells overexpressing PGC-1α enhance early skeletal muscle tissue formation

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Running header: Engineered hMPC_PGC-1α: faster muscle formation

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Abstract:

Muscle precursor cells (MPCs) are activated satellite cells capable of muscle fiber reconstruction. Therefore, autologous MPC transplantation is envisioned for the treatment of muscle diseases. However, the density of MPCs, as well as their proliferation and differentiation potential gradually decline with age. The goal of this research was to genetically modify human MPCs (hMPCs) to overexpress the peroxisome proliferator-activated receptor gamma coactivator (PGC-1α), a key regulator of exercise-mediated adaptation, and thereby to enhance early skeletal muscle formation and quality. We were able to confirm the sustained myogenic phenotype of the genetically modified hMPCs. While maintaining their viability and proliferation potential, PGC-1α modified hMPCs showed an enhanced myofiber formation capacity *in vitro*. Engineered muscle tissues were harvested 1, 2 and 4 weeks after subcutaneous injection of cell-collagen suspensions and histological analysis confirmed the earlier myotube formation in PGC-1α modified samples, predominantly of slow twitch myofibers. Increased contractile protein levels were detected by Western Blot. In summary, by genetically modifying hMPCs to overexpress PGC-1α we were able to promote early muscle fiber formation *in vitro* and *in vivo*, with an initial switch to slow type myofibers. Therefore, overexpressing PGC-1α is novel strategy to further enhance skeletal muscle tissue engineering.

Key Words: human muscle precursor cells, differentiation, skeletal muscle tissue engineering, PGC-1α

INTRODUCTION

Damage or loss of skeletal muscles is a major concern in many diseases. Autologous stem cell therapy is on the door step to successful clinical application and represents a novel treatment option for various muscle-related pathologies, including urinary incontinence[1], vocal cord dysfunction[2] and vesicoureteral reflux[3]. Satellite cells are quiescent cells, residing underneath the basal lamina of skeletal muscle fibers. They get activated after muscle tissue injury, turning into proliferating myoblasts, or muscle precursor cells (MPC). MPCs grant sufficient progeny for tissue repair[4,5]. Due to their potential to form new contractile myotubes, these cells are being investigated for muscle tissue engineering and reconstruction in the treatment of a variety of muscle disorders[6-9].

Despite recent progress in the field of muscle tissue bioengineering, a decreased proliferative capacity of MPCs due to donor age remains the main shortcoming of this approach[10-12]. This challenge for autologous cell therapy may be addressed by exercise and/or therapeutic regulation of gene expression, which enhances the ability of MPCs to restore muscle fibers. The transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a key player in neuromuscular activity of skeletal muscle and regulates important exercise-mediated adaptations[13,14]. The PGC-1α expression in a muscle is proportional to the amount of exercise and protects skeletal muscle cells from atrophy, thereby, being beneficial for the cell survival[15]. It serves as a transcriptional coactivator of nuclear receptors and transcription factors, that play essential roles in the regulation of cellular differentiation, development and metabolism (carbohydrate, lipid, protein) of higher organisms[15,16]. Hence, PGC-1α is regulating the

mitochondrial biogenesis and is adapting the oxidative state in muscles[14]. The increase of PGC-1α levels goes along with a shift in the myofiber composition toward high-endurance muscle fibers, that are capable of sustaining long-term contractions[17]. Slow-twitch myofibers mostly use oxygen as electron acceptor to generate energy (ATP), which is gradually released for sustained, long contractions, resistant to fatigue[18]. Additionally, this high endurance muscle phenotype is characterized with pronounced tissue vascularization, increased mitochondria and myoglobin levels and enhanced import of glucose, lipids and lactate[18]. Similar changes are observed in adaptation to exercise, a process also mediated by PGC-1α. The therapeutic potential of PGC-1α in a metabolic optimization strategy is currently being investigated and highlights the importance of novel approaches for successful muscle tissue engineering[19].

Muscle reconstruction using human MPCs (hMPCs) is a promising and feasible therapy method. However, further improvements towards engineering of larger muscles, functional myofiber formation and increased integration into the host tissue are needed. Our project thus aimed at the generation and validation of a viral vector for ectopic expression of PGC-1α in hMPCs, in order to assess the therapeutic potential of *in vivo* bioengineering of skeletal muscle tissue in a mouse model. We hypothesize that initial overexpression of PGC-1α would improve the muscle cell survival, myofiber formation capacity and expression of muscle-specific proteins in the hMPCs. Moreover, it would induce a myofiber type switch into slow-type myofibers, which are highly desirable for sphincter muscle bioengineering.

MATERIALS AND METHODS

Isolation and expansion of hMPCs

Human muscle biopsies from the *M. rectus abdominis* were randomly collected upon ethical approval by the local swiss ethics institutions and after written informed consent of hospitalized patients undergoing abdominal surgery under general anesthesia. The samples were processed according to established protocols[20]. Briefly, each muscle biopsy was first minced and digested with collagenase Type I 0.2% (w/v) (Sigma) and dispase 0.4% (w/v) (Gibco). The enzymatic reaction was terminated with medium containing 10% FBS. Individual myofibers were then liberated by rigorous pipetting and filtered through a strainer with a pore size of 100 µm. After centrifugation the pellet was resuspended in culture medium and the pieces of muscle fibers and dissociated cells transferred into 35 mm dishes coated with collagen type I (1mg/ml) (BD) as a pre-plating step. The culture medium consisted of DMEM/F12, 1% Penicillin/Streptomycin, 18% FBS, 10 ng/ml hEGF (Sigma), 1 ng/ml hbFGF (Sigma), 10 µg/ml human insulin (Sigma) and 0.4 µg/ml dexamethasone (0.5 µM, Sigma) [20]. After 24 h the supernatant containing non-adhered hMPCs was re-plated into dishes coated with collagen type I, in order to reduce the number of contaminating fibroblasts.

Adenoviral transduction

The AdEasy System was used as a tool for recombinant adenovirus generation. N-terminal HA-tagged human PGC-1α was cloned into an adenoviral vector that codes for CMV promoter-driven green fluorescent protein (GFP). The expression of hPGC-1α was also under the control of a CMV promoter, thereby ensuring its robust, constitutive expression. Successful cloning was validated by sequencing, while viral infection and

expression of the fluorescent reported genes was monitored by visualizing GFP (for the PGC-1α expressing adenoviral vector). As control for viral infection a GFP adenovirus was used. The viral titer was increased through additional amplification step and quantified by fluorescent and bright field microscopy. The optimal multiplicity of infection (MOI) was measured by serial titrations of the viral vectors on hMPCs and simultaneous determination of GFP expressing cells by fluorescent microscopy, cell toxicity and cell viability. An optimal transduction protocol in culture media was established beforehand showing at least 90% cell survival. Finally, the transduced hMPCs were expanded for 2 days after infection and were *s.c.* injected in nude mice, upon ethical approval by the local ethics committees.

Animal experimentation

All animal experiments were approved by the swiss animal ethics committee (Veterinäramt Zürich, Lic. Nr.: 12-2012) and performed according to the animal ethics welfare law. A total of 24 nude mice (8 weeks old, female, Charles River) were divided in two groups (GFP and PGC-1α). For the *in vivo* experiments, the hMPCs were expanded to passage 3-4. Each sample contained $30x10^6$ transduced hMPCs, which were gently mixed with 500 µl collagen type I carrier (final concentration: 2 mg/ml) (BD) and prepared for subcutaneous (*s.c.*) injection in the back of nude mice [21]. Each animal received two bilateral *s.c.* injections each containing $30x10^6$ hMPCs under general isoflurane anaesthesia and the engineered tissues were harvested after 1, 2, and 4 weeks.

Cell characterization

After expansion of hMPCs to passage 3 they were infected with the corresponding adenovirus and cultured for 2 days. The cells were characterized by fluorescence-activated cell sorting (FACS), using the following primary antibodies: anti-MyoD (1:100, BD), anti-MyHC (1:1, DSHB), anti-desmin (1:50, Sigma), anti-sarcomeric alpha-actinin (1:200, Sigma), anti-CD34 (1:100, BD) and anti-IgG Isotype control (1:100, Santa Cruise). Cy3 anti-mouse IgG (1:1000, Sigma) antibody was used as secondary antibody. A total of 50'000 events were registered by BD FACS Canto flow cytometer (BD Biosciences) immediately after labeling and the analysis was performed using FlowJo software v. 7.5 (Tree Star Inc.). All data are expressed as percentage of maximum (% Max). All measurements were performed with at least 3 different human biopsies.

Additionally, the transduced hMPCs were cultured to 70-80% confluency, fixed with 4% PFA for 10 min at room temperature, permeabilized with 0.5% TritonX-100 (Sigma) for 7 min, blocked for 30 min (5% BSA + 0.1% TritonX-100 in PBS), and finally stained with anti-desmin (1:50, Sigma), anti-sarcomeric α-actinin (1:200, Sigma), anti-CD34 (negative control) (1:100, BD) over night at 4 ^{o}C. After washing with PBS, the cells were incubated with Cy3 anti-mouse IgG secondary antibody (1:1000, Sigma) and DAPI (1:100, Sigma) for 1h at room temperature, washed again and finally mounted (Dako). Images were acquired with Leica-Imager Type DM6000B at exposures normalized to unstained controls (secondary antibody and DAPI only).

Fiber formation assay

Differentiation of hMPCs into myofibers *in vitro* was performed as previously reported (fiber formation assay)[20]. Briefly, hMPCs were grown to 50% confluency in culture medium and afterwards in differentiation medium (10% FBS) for 7-10 days until myofibers formed. The myofibers were fixed with ice-cold methanol (7 min), stained with Giemsa (1:20, 45-60 min) and air-dried. Five high-power-fields (HPF, 10x) were obtained per condition from cell samples from 4 patients` biopsies and the results were expressed as number of myofibers/HPF, number of nuclei/myofiber, and number of nuclei/HPF. The fusion rate was calculated by dividing the number of nuclei/myofiber by the number of nuclei/HPF (%). A total n=4x5HPF (20x) were analyzed. The imaging software "ImageJ for microscopy" was used for data assessment.

Cell viability and proliferation

In all cases, cell numbers and viability were confirmed by trypan blue staining after trypsinization. To evaluate proliferation and viability of the infected cells at different time points, hMPCs were cultured for 6 days. The cell proliferation reagent WST-1 (Roche) was used according to the manufacturer's protocol. For further confirmation of cell viability hMPCs were stained with 10 µM CellTrace Calcein Red-Orange, AM (Life Technologies) for 30 min at 37 °C. Viable cells were detected using a fluorescence microscope. All measurements were performed in duplicates of at least 3 different human biopsies.

Immuno-/Histological assessment

The harvested neoformed graft-derived tissues were embedded in cryo-preservative (OCT embedding medium, Cell Path) immediately after isolation. Cryostat sections were prepared (10 μm) and further processed. Haematoxylin and eosin (H&E) (Sigma) staining was performed according to the manufacturer's protocol. For immunohistological analysis the tissues were fixed with ice-cold MeOH (60 min), permeabilized (0.5% TritonX-100, 20 min), blocked for 30 min (5% BSA + 0.1% TritonX-100 in PBS), and finally stained with anti-MyHC (1:2, DSHB) over night at 4 ^{o}C. After washing with PBS, the tissues were incubated with Cy3 anti-mouse IgG secondary antibody (1:1000, Sigma) and DAPI (1:100, Sigma) for 1 h at room temperature, washed again and finally mounted (Dako). Images were acquired with Leica-Imager Type DM6000B at exposures normalized to unstained controls (secondary antibody and DAPI only).

Real-time PCR and CK assay

For analysis of PGC-1α downstream regulated genes (by RTPCR) and creatine kinase (CK) levels (measured using the Cobas c111 system (Roche Diagnostics) according to manufacturer's protocol), the cells were cultured for 2 days after infection and then transferred to differentiation medium for 9 h, or until day 6, respectively, and finally harvested for further assessments. For gene analysis of tissue, the harvested tissues were pulverized in liquid nitrogen and suspended in RNA lysis buffer. Total RNA was isolated for both, cells and tissues, using the SV Total RNA Isolation System kit

(Promega) according to the manufacturer's protocol, which includes a DNase digestion. RNA was reverse transcribed with random primers (High-Capacity cDNA reverse transcription, Life Technologies). Pre-designed primers for human PPARGC1 (Hs01016719_m1), MYH1 (Hs00428600_m1), desmin (Hs00157258_m1) and VEGF (Hs00900055_m1) were purchased from Life Technologies. Further primers were purchased from Microsynth: hCox5b (fwd primer: ATG GCT TCA AGG TTA CTT CGC, rev primer: CCC TTT GGG GCC AGT ACA TT), hCycS (fwd primer: CTT TGG GCG GAA GAC AGG TC, rev primer: TTA TTG GCG GCT GTG TAA GAG), hERRα (fwd primer: AGG GTT CCT CGG AGA CAG AG, rev primer: TCA CAG GAT GCC ACA CCA TAG), hPGC-1α (fwd primer: TCT GAG TCT GTA TGG AGT GAC AT, rev primer: CCA AGT CGT TCA CAT CTA GTT CA), and hTBP (fwd primer: CCC GAA ACG CCG AAT ATA ATC C, rev primer: AAT CAG TGC CGT GGT TCG TG). 18S rRNA (4319413E) was used to normalize cDNA concentrations. For quantification, the expression of each gene was normalized to the 18S, or TBP expression in the corresponding sample. The entire experiment was repeated at least three times and samples were analyzed in triplicates.

Gel electrophoresis and immunoblotting

In summary, tissues were pulverized in liquid nitrogen with a mortar/pestle and suspended in lysis buffer supplemented with a protease inhibitor cocktail (Sigma). Afterwards, the samples were centrifuged for 20 min at 13,000 rpm, and the supernatant was collected for protein determination. The total protein was measured with the BCA Protein Assay Kit (Thermo Scientific) and protein lysate (30-50 µg) was loaded on a

10% or 12% gel (Bio-Rad). Western blot was performed according to the manufacturer's protocol. After the separated proteins were electro-transferred onto PVDF membrane (Immobilion-P; Millipore), the latter was incubated with a primary antibody at 4 °C overnight in TBS, 0.1% Tween-20 and 5% non-fat dry milk. The primary antibodies used were anti-MyHC1 (1:5, DSHB), anti-MyHC (1:1, DSHB), anti-desmin (1:50, Sigma), anti-PGC-1α (1:1000, Calbiochem), anti-sarcomeric α-actinin (1:2000, Sigma), anti-GAPDH (1:2500, Sigma), and α-tubulin (1:2000, Bioconcept). Finally, the membranes were washed in TBS with 0.1% Tween-20 for 30 min and incubated with the appropriate HRP-conjugated secondary antibody (Amersham Pharmacia Biotech) for 1 h. The signals were detected by the ECL method (ECL-Kit, Amersham). The data were analyzed by Image Studio Lite (Li-Cor) software and represented as protein expression relative to GAPDH.

Statistics

For statistical analysis IBM SPSS v22.0 (SPSS Inc,) was used and graphics were drawn with GraphPad Prism v5.04 (GraphPad Software, Inc.). All data were analyzed by Student's *t*-tests for paired samples or one-way ANOVA with Bonferroni or LSD post-hoc analysis ($p<0.05$ was considered significant). All presented data are expressed as means with corresponding standard error of the mean (±SEM).

RESULTS

Generation and establishment of genetically modified hMPC

Adenoviral constructs containing hPGC-1α, or GFP only, were successfully generated and amplified for further use in hMPCs. The expression of each gene was designed to be under the control of a CMV promoter in order to assure robust expression of the transgene. The transduction efficiency was confirmed by fluorescent imaging, as both constructs contain a green fluorophore, expressed under a separate CMV promoter (Fig.1. A, green). The constructs enabled detection of transduced cells both prior to transplantation for assessing the cell transduction efficiency, and afterwards, on engineered muscle sections. The safety of the viral infection was visualized by a cell viability assay (CaAM) (Fig. 1 A, red) and further confirmed by Trypan-blue staining for distinction of live/dead cells (Fig. 1 B), showing no significant differences between infected (GFP (90.5±1.97, n=6), PGC-1α (88.16±5.32, n=6)) and wild type (WT, 89.00±3.01, n=8) cells. The proliferation capacity of the WT, GFP- and PGC-1α-overexpressing hMPCs was determined by a cell proliferation assay (WST-1) over 6 days culturing after transduction, showing no significant variations between the different groups (Fig. 1 C, at 6 days: WT (2.77±0.18, n=6); GFP (3.12±0.19, n=9); PGC-1α (2.59±0.29, n=12)). Importantly, the designed adenoviruses did not affect the expression of specific muscle protein markers (sarcomeric α-actinin and desmin), as detected by immunofluorescence imaging of hMPCs (Fig. 2 A) and FACS (Fig. 2 B) under culturing conditions. The latter also showed expression of specific well-described markers for characterization of activated hMPCs[4], as well as a significant shift in GFP expression levels in both transgenic groups, compared to uninfected WT cells (Fig. 2

C). These results confirm the successful adenoviral transduction of hMPCs and their maintained cell phenotype.

PGC-1α overexpression facilitates differentiation of hMPCs into myotubes *in vitro*

To directly assess the role of PGC-1α in myofiber formation, an *in vitro* hMPCs differentiation experiment was performed. A Fiber Formation Assay[22] with untransduced (WT), GFP- and PGC-1α-infected cells revealed an increased fusion rate in PGC-1α overexpressing myoblasts (Fig. 3 A and B, n=4x5 HPF). The participation of transduced GFP-positive cells in the myotube formation could be visualized by fluorescent microscopy (Fig. 3 A, second row, fluorescence). The facilitated initial differentiation of the PGC-1α overexpressing cells was further confirmed by Western Blot (Fig. 3 C) where elevated desmin levels were detected already at day 2 after infection after 9h of differentiation.

The induction of differentiation goes hand in hand with an increase in metabolic activity (ATP consumption). To analyze this process, the intra- and extra-cellular creatine kinase (iCK and eCK) levels were measured 2 and 6 days after adenoviral infection and initiation of differentiation The iCK levels were significantly increased in PGC-1α infected cells at day 2 compared to GFP-infected cells ($p<0.001$) (Fig. 3 D). At day 6 an overall increase in iCK levels in all 3 groups, concomitant with the induced differentiation could be seen (Fig. 3 D). Importantly, an increase in eCK levels is often associated with muscle damage, cell death and membrane disruption[23]. Our results show that the viral infection did not affect the viability and integrity of hMPCs and that the eCK levels remained low 2 and 6 days after infection (Fig. 3 E).

Efficient elevation of PGC-1α and downstream regulated genes using viral vectors in hMPCs

Functionality of the expressed PGC-1α protein was validated by determination of well-described target genes of the coactivator in muscle cells. As expected, the introduction of the PGC-1α viral vector specifically elevated the corresponding gene expression level after 2 days ($p<0.01$) and 6 days ($p<0.0001$) after inducing cell differentiation (Fig. 3 F and G). The bioactivity of the construct was furthermore confirmed after 2 and 6 days, respectively, by the induction of its target genes cytochrome c oxidase subunit 5b (Cox5b) ($p<0.05$, $p<0.01$), cytochrome c (CycS) ($p<0.001$, $p<0.001$) and estrogen-related receptor α (ERR α) ($p<0.0001$, $p<0.05$), compared to the control cells infected with GFP and untransduced hMPCs (WT) (Fig. 3 H and I). These data confirm the efficiency of the presented PGC-1α adenoviral construct for genetic modification of hMPCs.

PGC-1α triggers early myotube formation *in vivo*

Encouraged by our *in vitro* observations, we further evaluated the capability of transduced hMPCs to form ectopic muscle tissue *in vivo*. The tissues formed by subcutaneously injected hMPCs (transduced with GFP or PGC-1α) were harvested after 1, 2 and 4 weeks for analysis. The weight of the collected tissues did not differ significantly between the two groups (GFP and PGC-1α: 114.9±18.73mg, 88.47±15.96mg ($p=0.3021$); 39±5.84 mg, 42.33±4.76mg ($p=0.6814$), 19.44±2.33mg, 22.94±2.28mg ($p=0.3219$) at 1, 2 and 4 weeks, respectively, data not shown), although a

tendency for visually smaller PGC-1α tissues at 1 week was observed, concomitant with the enhanced differentiation (Fig. 4 A). The constant tissue volume decrease over time in the *ex situ* model due to myofiber formation and simultaneous collagen remodeling has previously been reported[21]. Histological assessment via H&E staining of the engineered tissue revealed earlier formation of myotubes in the PGC-1α samples (already at week 1) (Fig. 4 C, black arrows). This was further confirmed by immunohistological staining of the samples for MyHC, a marker for muscle differentiation and maturation, displaying an increased signal (Cy3, red) in the PGC-1α transduced tissues and more organized structures (myotubes) (Fig. 4 D, white arrowheads). The typical red color of slow twitch oxidative type I myofibers [17] could be detected macroscopically after tissue pulverization with liquid nitrogen in the PGC-1α samples, but not in the control GFP samples after 1 week (Fig. 4 B). This was concomitant with an initial significant increase in VEGF-A gene levels ($p<0.0001$) in the harvested PGC-1α overexpressing tissues (Fig. 5 A). The PGC-1α overexpression was confirmed by RTPCR (Fig. 5 B, $p=0.009$). The earlier differentiation was further confirmed by an increase in desmin ($p=0.047$) and MyHC1 ($p=0.024$) gene expression in the PGC-1α infected samples, while GFP-infected samples did not show expression at week 1 (Fig. 5 C and D, n=3-5). In line with this results, at protein level, PGC-1α overexpressing engineered muscle tissue also indicated an increase in relative expression levels of desmin (Fig. 5 E, 2.36±1.32, n=6) and MyHC1 (Fig. 5 F, 2.24±0.67, n=4) at week 1, compared to GFP samples. An increase in the expression of MyHC protein over time could be observed (Fig. 5 G, 2.5±0.96 at 4.week, n=4), leading to a suggestion for a PGC-1α-induced shift towards MyHC1 type myofibers at 1 week

(Fig. 5 H). Based on the findings above, PGC-1α overexpressing hMPCs hold promise for the enhanced repair of skeletal muscle tissue due to their capacity to speed up myofiber formation, with an initial shift to oxidative type I myofibers.

Discussion

Muscle tissue bioengineering has made substantial progress over the last decade, allowing us to grow functional muscle tissue[21,24]. Subcutaneous implantation of myoblasts may have a range of useful applications, from the study of myogenesis to the delivery of gene products[24]. Nevertheless, there are still substantial limitations in size and quality of the engineered constructs. Additionally, drugs including testosterone, growth hormone, leptin, myostatin inhibitors, creatine and vitamin D have been successfully used to support muscle strength and growth[25]. Yet, there are safety concerns for their long-term application and, therefore, more investigations are required in order to minimize their adverse effects. Another possible solution for rebuilding muscle tissues is cell therapy using satellite cells. The induced expression of several factors in MPCs has extensively been studied, mostly to obtain enhanced cell viability and proliferation, or to prevent apoptosis and induce angiogenesis, or to induce gap-junction formation against cardiac arrhythmias[26].

Many muscle disorders are associated with skeletal muscle inactivity and exercise is an exceptional therapeutic mediation for many disease conditions[27]. Although the exact mechanisms mediating these healing effects still remain elusive, several pathways have been proposed[28]. While resistance training combined with adequate nutrition remains the most effective intervention for diminishing the functional decline in muscles, there is a certain age-linked barrier to obtaining full benefits from this therapy[25].

A promising molecule controlling skeletal muscle metabolism with potential therapeutic effect has been identified[14]. PGC-1α is a known and potent transcription coactivator for nuclear receptors and other transcription factors. It is expressed in skeletal muscles and is a powerful master regulator of mitochondrial bio-genesis[29]. Interestingly, PGC-1α has been shown to increase the expression of myofiber type I fibrillar proteins by co-activating the myocyte enhancer factor 2 (Mef2) transcription factors, thereby coordinating the expression of both metabolic and contractile properties of type I myofibers[17]. These findings make PGC-1α a good candidate for targeted regulation of skeletal muscle cell metabolism and plasticity in the myofiber formation process, and, therefore, we decided to research its applicability for the bioengineering of improved muscle tissues.

The ultimate goal in skeletal muscle tissue engineering is the construction of a functional tissue of a desired myofiber type. Interested in sphincter muscle bioengineering, we aimed at improving the regenerative potential of human MPCs by inducing PGC-1α overexpression through adenoviral gene delivery. By overexpressing PGC-1α in hMPCs we targeted a major crossroad of intracellular pathways, affecting cell metabolism (mitochondrial activity upregulation) and, simultaneously, enhancing the early expression of contractile proteins (MyHC1, desmin) and secretory factors (VEGF-A), thereby facilitating the process of skeletal muscle bioengineering. Our data showed feasibility of viral overexpression of PGC-1α with desired phenotypic changes in the ectopic muscle but importantly without detrimental side effects on hMPC viability and differentiation *in vivo* and *in vitro*. We acknowledge that WST-1 measurement is dependent upon mitochondrial function and PGC-1α might mask the

possible lack of proliferation in the infected cells. However, we visualized the cell viability by CaAM assay and quantified it by trypan blue staining, without observing any detrimental effects upon PGC-1α viral infection.

The bioactivity of PGC-1α was confirmed by significantly enhanced expression of PGC-1α downstream targets, proving the functional efficiency of the presented construct. Some supporting evidence suggests an increased expression of mitochondrial and other metabolic genes as a plausible mechanism for rescuing a damaged muscle[27]. Therefore, the demonstrated increase in PGC-1α downstream mitochondrial targets was a key milestone for further *in vitro* and *in vivo* studies.

A crucial role in the process of forming new muscle tissue is played by the capacity of myoblasts to differentiate into myotubes/myofibers and a sustained energy metabolism. Our genetically modified hMPCs showed a significantly increased fusion rate, in parallel with an enhanced energy metabolism, depicted by iCK levels and mitochondrial gene level analysis. Given the low iCK levels in myoblasts and their increase with myotube formation, our results indicate that viral overexpression of PGC-1α drives higher iCK expression, possibly due to a faster differentiation process. Moreover, elevated desmin protein expression was detected 2 days after the adenoviral infection, showing the early appearance of sarcomeric architecture. Consistent with the observed facilitated *in vitro* differentiation, the ectopically formed skeletal muscle tissue with PGC-1α overexpression revealed myotube formation and increased expression of contractile proteins (desmin and MyHC1) already after 1 week, indicating facilitated differentiation. Interestingly, this strong effect was reduced at later time points. However, PGC-1α modified samples expressed higher levels of general MyHC with an

increase over time, when compared to GFP samples, while there was higher MyHC1 (slow twitch) protein expression at one week. These data indicate a possible initial shift towards slow type myofibers. Related gene-delivery studies suggest that overexpression of certain factors (e.g. VEGF) may be beneficial at the time of cell transplantation, and their transient effect may prevent the risk of malignancy formations[30,31].

Vascularization of the bioengineered tissue is the bottle-neck of many approaches. Importantly, PGC-1α overexpressing tissues showed enhanced VEGF-A gene levels. Secretion of various effectors by the injected hMPCs may also contribute to optimization of the regenerative process (e.g. increased neovascularization)[21,32]. Moreover, PGC-1α has been demonstrated to stimulate angiogenesis in skeletal muscle by inducing the release of key factors, including VEGF, in cultured muscle cells and skeletal muscle *in vivo*[33]. Furthermore, PGC-1α seems to have led to overexpression of VEGF-A, thereby, probably inducing early neo-vascularization and counteracting necrosis after implantation, possibly leading to improved cell/tissue survival and better long term survival. In line with our *in vivo* observations, others have shown, that an increased VEGF release in a hypoxic environment leads to enhanced differentiation[34]. This could explain the observed increase in contractile MyHC protein and myofiber formation in PGC-1α engineered tissues, when compared to the corresponding GFP samples.

Whether the accelerated differentiation seen in this research using an *ex situ* model really leads to functional improvement during regeneration has to be validated using *i.m.* injection of the modified cells. However, the use of ectopic model for muscle tissue engineering also brings along valuable information about the formation of muscle fibers

in a non-myogenic environment, as it might be in the patients, e.g. scar tissue. Considering these facts, the optimal model to study skeletal muscle regeneration would be to inject the cells in injured muscles. Further, the use of viral gene delivery for clinical applications is still associated with several drawbacks[35]. We acknowledge that the choice of the adenoviral delivery method might not be the most suitable one. For a clinical set up, we would suggest a non-viral gene delivery method. We further acknowledge a possible immune reaction from the mice we chose, although no detrimental effects were detected in the engineered tissues. Still this model sets a significant milestone towards the possible engineering of improved muscle tissue of a desired myofiber type.

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The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. PGC-1α adenoviral infection without adverse effects on hMPC. The successful adenoviral transduction of hMPCs with GFP and PGC-1α (A, green) did not affect the cell viability, as shown by CaAM assay (A, red). This was further confirmed by Trypan Blue staining of live/dead cells (B). Proliferation rate also did not differ between the wild type (WT) non-infected cells and the infected hMPCs (C). Scale bar: 50μm

Figure 2. Adenoviral infection did not change the hMPC phenotype. The expression of typical muscle markers (Cy3-red) in the infected cells (green) was confirmed by Immunocytochemistry. Nuclei were stained with DAPI (blue) (A). The hMPCs were analyzed by FACS for expression of various muscle marker proteins (B) and the successful transduction was confirmed by a shift in FITC signal (C). Scale bar: 25μm

Figure 3. PGC-1α overexpressing hMPCs differentiate faster into myotubes *in vitro*. Fiber formation assay [22] and fluorescent microscopy revealed successful myotube formation in WT hMPCs and infected cells (green fluorescence) respectively (A), with an increased fusion rate in PGC-1α overexpressing cells (B). Two days after infection, PGC-1α overexpressing hMPCs displayed enhanced desmin protein levels (C). Intracellular CK (iCK) levels were significantly increased 2 days after PGC-1α infection (D), whereas extracellular (eCK) levels remained unaffected (E). An overall increase in iCK levels was observed 6 days after inducing differentiation of the hMPCs (D) and eCK remained unchanged (E). PGC-1α gene expression levels were upregulated in hMPCs, infected with PGC-1α adenovirus after 2 (F) and 6 days (G) post infection, compared to GFP and WT controls. PGC-1α downstream genes (hCox5b; hCycS;

hERRα) were upregulated in hMPCs transduced with PGC-1α, respectively (H, I). Data are presented as gene expression relative to hTBP, using the ddCt method. Scale bar: 50μm

Figure 4. Engineered muscle formation is enhanced by overexpression of PGC-1α in hMPCs. hMPCs transduced with PGC-1α, or control GFP adenovirus were injected subcutaneously on both sides of the back of nude mice. The harvested tissue was visualized macroscopically at 1 week at the subcutaneous tissue (A). The typical red color of oxidative type I myofibers was enriched in PGC-1α samples, compared to GFP controls (samples powderized with liquid nitrogen) (B). The process of successful myofiber formation was visualized over 4 weeks with H&E staining (C), revealing an increased early myotube formation in PGC-1α overexpressing samples (black arrows). *Tibialis anterior* (TA) was used as native control for muscle fiber formation. The increased differentiation capacity of PGC-1α transgenic hMPCs at early time points (1 week) was also envisioned by immunostaining for MyHC (Cy3, red) of the *ex situ* engineered muscle fibers (green) (DAPI, blue) (D). White scale bar: 50 μm, black scale bar: 0.5cm

Figure 5. Gene and protein expression in *ex situ* bioengineered muscle tissues, overexpressing PGC-1α. Gradual increase of VEGF-A gene expression levels was shown by RTPCR (A). PGC-1α gene overexpression was confirmed (B). Desmin and MyHC1 gene expression levels (relative to 18S) were increased in PGC-1α overexpressing tissues (C, D). The enhanced differentiation in PGC-1α overexpressing tissues could also be shown by increased contractile protein expression levels in the

corresponding bioengineered tissues (E-G). The ratio of MyHC1 to general MyHC showed an initial switch to oxidative type I myofibers (H).

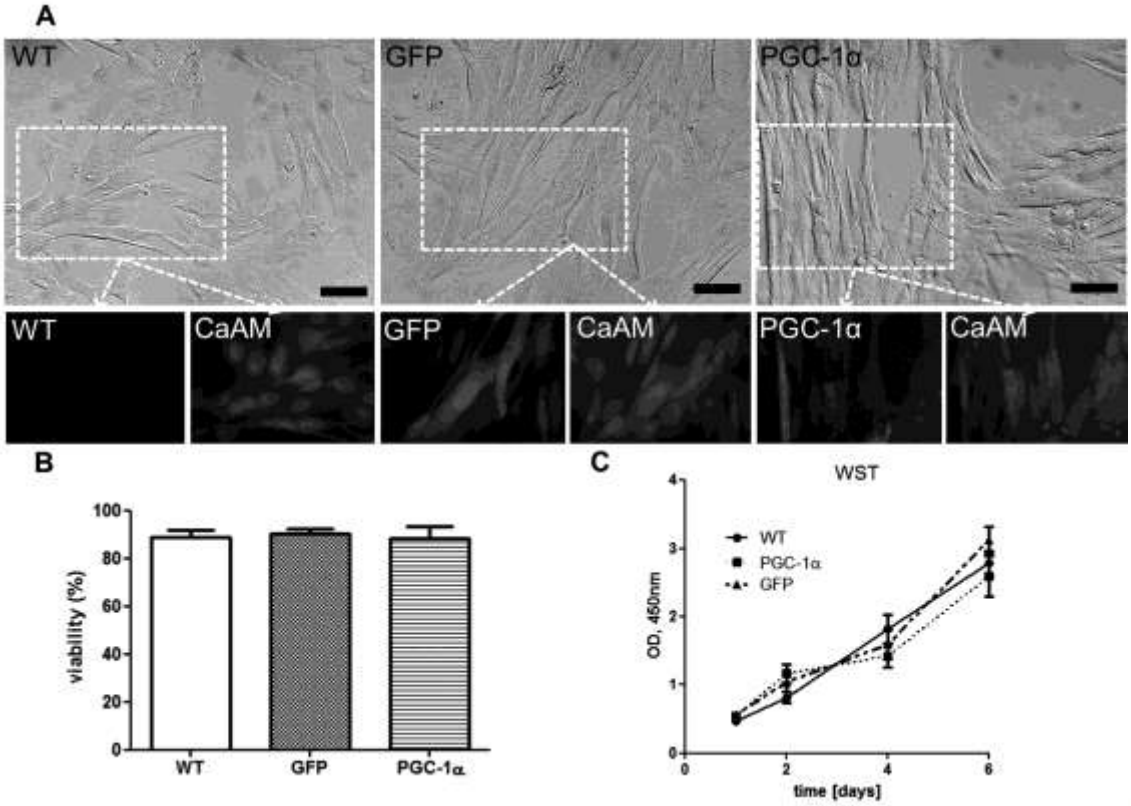

Figure 1

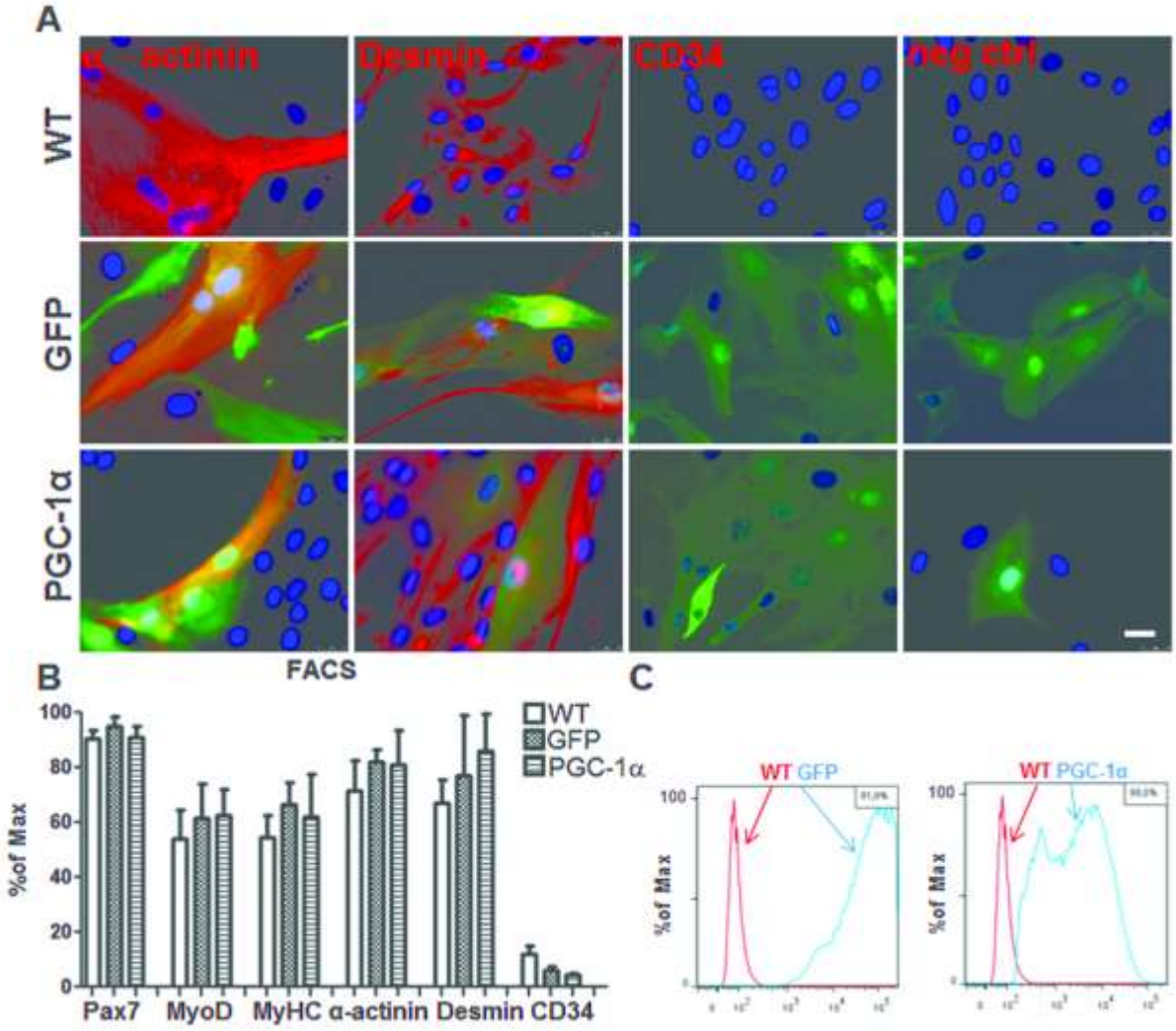

Figure 2